PHOSPHATASE ACTIVITY IN TETRAHYMENA [1]

ALFRED M. ELLIOTT AND ROBERT L. HUNTER [2]

Department of Zoology, University of Michigan, and Marine Biological Laboratory, Woods Hole, Massachusetts

Histochemical studies by Weisz (1949) with normal and re-organizing Stentors and those by Sullivan (1950) with *Colpidium campylum* have demonstrated the presence of phosphatases in Protozoa. The present investigation was undertaken to increase our knowledge concerning the action of these enzymes in the ciliate protozoan, *Tetrahymena geleii,* and to correlate this activity with growth.

Materials and Methods

The organism employed in this investigation, strain E, was maintained in 250 ml. Erlenmeyer flasks under aseptic conditions except where sterility was not necessary. The media were prepared with double glass-distilled water and were sterilized in the autoclave 10 minutes at 15 pounds pressure. The pH was adjusted to 7.4 with 1/*N* NaOH before sterilization except where pH was specifically under investigation. All cultures were incubated at 24° in the dark. The numbers of organisms were estimated with the Klett-Summerson photoelectric colorimeter according to a method reported earlier (Elliott, 1949b).

Analyses for total and inorganic phosphorus were performed with the methods of Fiske and Subbarow (1925). At stated intervals 6-ml. samples were withdrawn aseptically with pipettes and the organisms separated from the supernatant by centrifugation (100 times gravity for 2 minutes). Phosphorus determinations were made on 2-ml. aliquots of the supernatant. The organisms were washed twice and then re-suspended in distilled water for estimating numbers in the colorimeter. Finally, where indicated, total phosphorus determinations were made on 2-ml. aliquots of the organisms. All experiments were repeated several times and the most representative selected for reporting here.

Experimental

Presence of phosphatases

In order to demonstrate the presence of phosphatases (nucleotidases) in Tetrahymena, a heavy suspension of the cells (approximately 20,000 cells/ml.), taken from proteose-peptone medium during the logarithmic growth phase, was washed twice in distilled water and then suspended in a 0.0005 *M* solution of the nucleotide,

[1] This investigation was supported in part by a grant from the Horace H. Rackham School of Graduate Studies. The authors are grateful to Merck and Company for supplying the amino acids and vitamins used in this investigation, and to Dr. E. L. R. Stockstad of the Lederle Laboratories for furnishing the protogen, without which these experiments could not have been carried out.

[2] Teaching Fellow, Department of Zoology, University of Michigan.

adenylic acid. For convenience the rigid sterile technic used in the remainder of the experiments was somewhat relaxed because it was felt that the combined effects of deficient medium and bacterial predation by the ciliate were adequate to control the effect caused by any slight contamination that might occur. The pH was adjusted electrometrically (Beckman) to 7.4 with 1/*N* NaOH. Final pH determinations indicated a slight drop, but not below 7.1. Inorganic phosphorus determinations were made at intervals for 50 hours of incubation and are recorded in Figure 1.

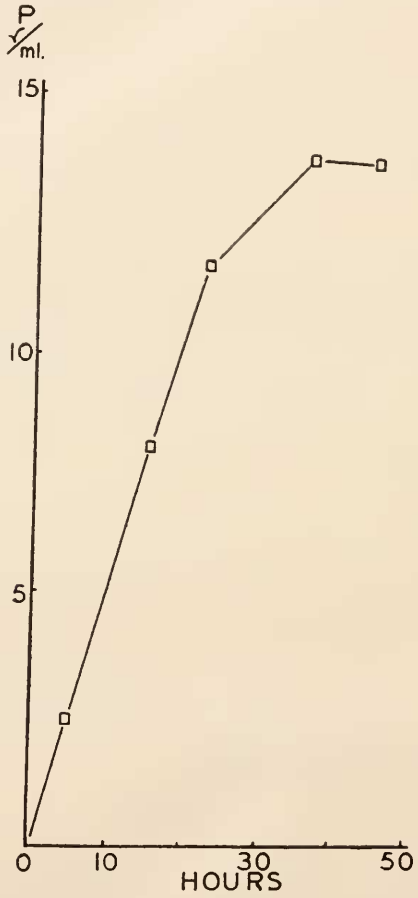

FIGURE 1. Amount of phosphate released plotted against time.

Over 80 per cent of the phosphate in adenylic acid was split off in the first 22 hours and the remaining 20 per cent was released in the next 13 hours. Parallel experiments with cytidylic acid demonstrated similar phosphate release, thus indicating the presence of active nucleotidases.

Location of the enzymes

The question as to whether the enzymes were confined to the cells or released into the surrounding medium was solved by repeating the above experiment, except

that after 14 hours of incubation one-half of the culture was filtered through a Seitz in order to remove the cells from that half; the amount of phosphate release was followed from that time in both flasks. The results are recorded in Figure 2. It is clear that as soon as the cells were removed from the culture, enzyme activity ceased. The enzymes are, therefore, confined to the cells and are not perceptibly released into the culture medium.

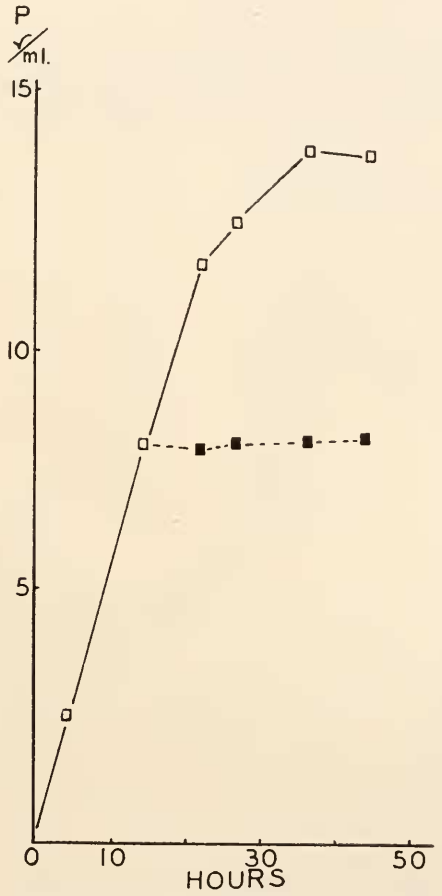

FIGURE 2. Same as Figure 1, except the flask was divided after 14 hours of incubation. Phosphate release indicated by black blocks.

Whether or not the nucleotide was taken into the cell and the phosphate released after being split intracellularly was the next point of interest. If the fate of the adenosine, which results from splitting phosphate from adenylic acid, could be followed, a still more precise location of the enzyme could be predicted. In order to check this point adenylic acid was again prepared as in the two preceding experiments, but this time the absorption of adenine at 260 mμ was followed through 48 hours of incubation, using a Beckman ultraviolet spectrophotometer. Five-ml. samples were removed from the flask at 6–12 hour intervals and the organisms

separated by centrifugation. Appropriate dilutions of the supernatant were made with $N/20$ NaOH and absorption determinations taken.[3] The results of this study indicated that the adenine level remained constant throughout the period of nucleotide breakdown. Apparently, then, the enzyme action takes place either within the cell or at the membrane and perhaps the value of this reaction is to obtain energy for cellular metabolism.

Relation to pH

The activity of the enzymes in relation to pH was demonstrated by following the inorganic phosphate release from adenylic acid at various pH levels. The results are recorded in Figure 3 where the organisms released the greatest amount

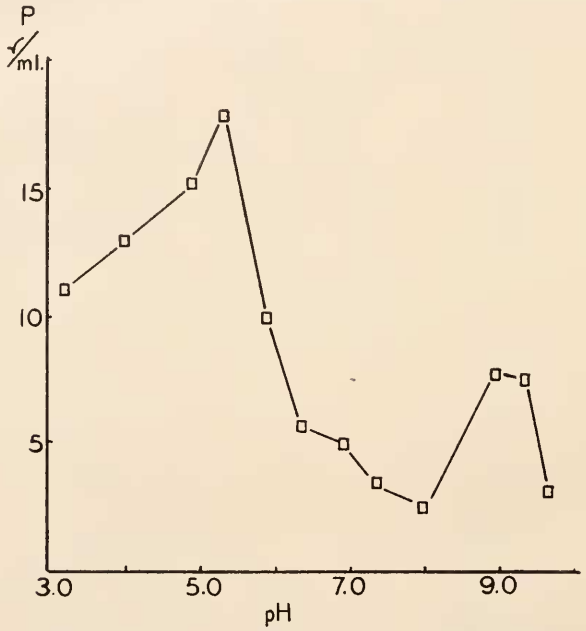

FIGURE 3. Phosphate release at various pH levels.

of phosphate at two levels, namely, 5.3 and 9.2. Since the system is complicated by intact cells in the medium, it is impossible to say that the nucleotidase activity is influenced by pH in the manner shown by these experiments, although it may be.

It might seem strange that all of the experiments were maintained at pH 7.4 rather than at the levels where enzyme activity in this system was optimal. It so happens that both of these levels are outside the range for optimal growth, and therefore might be injurious to the organism. There seems to be no correlation between pH levels which promote optimal phosphatase activity in living cells and those that support optimal growth of those cells.

[3] The adenine determinations were made through the courtesy of Dr. James E. Hogg, Department of Biological Chemistry, University of Michigan. The absorptions of adenine, adenylic acid and adenosine do not differ more than 3 per cent in $N/20$ NaOH solution (Morton, 1942).

Relation to growth

In order to determine phosphatase activity during the logarithmic growth phase of Tetrahymena, it was decided to follow both the release of inorganic phosphate and the phosphate uptake, as indicated by making total phosphorus determinations on both the supernatant and the cells themselves during the logarithmic growth period. The basal medium (Table I) was the same as that employed earlier

TABLE I

Basal medium

	Micrograms per milliliter		Micrograms per milliliter
l-Arginine	600	$FeCl_3 \cdot 6H_2O$	1
l-Histidine	150	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	5
dl-Isoleucine	75	$CuCl_2 \cdot 2H_2O$	5
l-Leucine	75	$MnCl_2 \cdot 4H_2O$	.05
l-Lysine	150	$ZnCl_2$	.05
dl-Methionine	150	Ca pantothenate	.10
dl-Phenylalanine	150	Nicotinamide	.10
dl-Serine	300	Pyridoxine	2.00
dl-Threonine	300	Riboflavin	0.10
l-Tryptophan	150	Pteroyltriglutamic acid	.01
dl-Valine	150	Thiamine	1.0
		Protogen	1.0 unit
Dextrose	1000		
Sodium acetate	1000	Adenylic acid	75
		Guanylic acid	75
$MgSO_4 \cdot 7H_2O$	100	Cytidylic acid	75
$CaCl_2 \cdot 2H_2O$	50	Uracil	75

(Elliott, 1949a, 1950) with the exception that no inorganic phosphate was included in the form of K_2HPO_4 and nucleic acid components were employed instead of yeast nucleic acid. The concentration of these components was raised from 100 to 300 γ/ml., which increased the growth rate by 50 per cent. The only source of inorganic phosphorus was that inadvertently introduced with protogen, an essential growth factor (Stokstad *et al.*, 1949), and this amounted to 2 γ/ml. Six-ml. samples were removed from the experimental flasks and the cells separated by centrifugation; 2 ml. of the supernatant was checked for inorganic phosphorus and another 2 ml. for total phosphorus. The cells were washed twice, then re-suspended in distilled water and numbers estimated in the colorimeter. A 2-ml. aliquot of this suspension was then taken for total phosphorus determinations of the organisms. From these data the curves in Figure 4 were constructed.

The total phosphorus in the supernatant declined at a rate which was the reciprocal of that taken up by the cells, as one might expect. These determinations account for about 95 per cent of the phosphorus. There is an indication that the total supernatant phosphorus continued to decline and the total phosphorus in the cells continued to increase following the growth peak; the reason for this is not clear. It may be that the cells store phosphate after they cease dividing.

The inorganic phosphorus showed the same explosive increase that was noted earlier. Approximately 75 per cent of the inorganic phosphorus is split off while the organisms are still in the lag phase of growth. Once the organisms enter the loga-

rithmic growth phase phosphate is utilized rapidly, hence the curve then shows a decline in the amount of inorganic phosphorus in the medium.

Discussion

Weisz (1949, p. 110), employing histochemical methods, was able to show acid phosphatases in normal Stentors "around the macronuclei, the basal granules of the

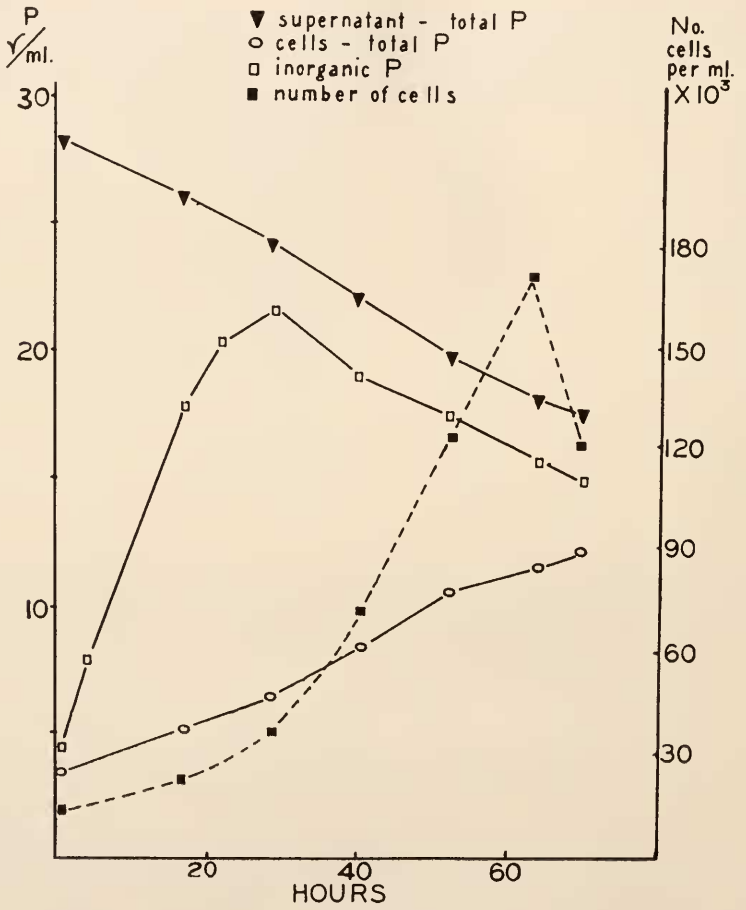

Figure 4. Relation of growth, phosphate release and phosphate uptake.

membranelles and the body cilia, in the endoplasmic fat vacuoles, and around the gastrioles." Sullivan (1950, p. 270), working with similar technics, localized alkaline phosphatase activity "in the perinuclear region of the cell." He used glycerophosphate as a substrate, which may mean that he is not dealing with the same enzymes that are evident in the present investigation. The present observations demonstrate the existence of nucleotidases in *T. geleii* (strain E), but their exact location within the cell cannot be determined from the data. If we are dealing with

the same enzymes as those found by Sullivan, one would expect that the nucleotide (adenylic acid) is absorbed into the perinuclear region of the cell where degradation takes place. This would also mean that the degraded fragments, inorganic phosphate and adenosine, would then be excreted into the medium. However, the present data on both inorganic phosphate and absorption determinations do not demonstrate the absorption of adenylic acid into the cell, only that the molecule is hydrolyzed which may take place at the membrane or intracellularly.

These observations indicate clearly that the enzymes are confined to the organism and are not elaborated into the surrounding medium, which might lend weight to the argument that the enzymes are intracellular. Tetrahymena does produce extracellular enzymes, however, as indicated by its ability to hydrolyze gelatin and casein (Elliott, 1933).

The active nature of these phosphatases in Tetrahymena may have some significance in its natural environment. This organism normally lives at the bottom of ponds where the organic material is undergoing rapid decomposition and where the concentration of inorganic phosphate is high. Numerous studies have shown that the source of this phosphate is in the thin layer of mud at the bottom of the pond or lake where there is a veritable microcosm of flora, fauna and other microorganisms (Einsele, 1938). Perhaps Tetrahymena, along with other microorganisms, aids in splitting off inorganic phosphate from organic debris and helps maintain the phosphate level in these bodies of water.

Summary

1. *Tetrahymena geleii E* possesses phosphatases (nucleotidases) capable of hydrolyzing nucleic acid and its components at a very rapid rate. Eighty per cent of the phosphate in 0.0005 *M* adenylic acid is released in 22 hours when the population level is 20,000 cells/ml.

2. The enzymes are confined to the cells and are not released into the surrounding medium.

3. The cells release phosphate most rapidly at two pH levels, 5.3 and 9.2, being most active in the acid range.

4. Enzyme activity as indicated by phosphate release is greatest in the lag phase of the growth cycle. During the logarithmic growth phase, the released phosphate is taken up by the cells at a uniform rate.

LITERATURE CITED

Einsele, Wilhelm, 1938. Über chemische und kolloidchemische Vorgänge in Eisen-Phosphat-Systemen unter limnochemischen und limnogeologischen Gesichtspunkten. *Arch. f. Hydrobiol.*, **33**: 361–387.

Elliott, A. M., 1933. Isolation of *Colpidium striatum* Stokes in bacteria-free medium and the relation of growth to pH of the medium. *Biol. Bull.*, **65**: 45–56.

Elliott, A. M., 1949a. The amino acid requirements of *Tetrahymena geleii E. Physiol. Zool.*, **22**: 337–345.

Elliott, A. M., 1949b. A photoelectric colorimeter for estimating protozoan population densities. *Trans. Am. Microscop. Soc.*, **68**: 228–233.

Elliott, A. M., 1950. The growth-factor requirements of *Tetrahymena geleii E. Physiol. Zool.*, **23**: 85–91.

Fiske, C. H., and Y. Subbarow, 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**: 375–400.

Morton, R. A., 1942. Application of absorption spectra to the study of vitamins, hormones and coenzymes. Adam Hilger, Ltd., London.

Stokstad, E. L. R., C. E. Hoffman, M. A. Regan, D. Fordham and T. H. Jukes, 1949. Observations on an unknown growth factor essential for *Tetrahymena geleii*. *Arch. Biochem.*, **20**: 75–82.

Sullivan, W. D., 1950. Distribution of alkaline phosphatase in *Colpidium campylum*. *Trans. Am. Microscop. Soc.*, **69**: 267–271.

Weisz, P. B., 1949. Phosphatases in normal and reorganizing Stentors. *Biol. Bull.*, **97**: 108–110.